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Transcriptional profiling of human gingival fibroblasts in response to multi-species *in vitro* subgingival biofilms

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Abstract

Periodontitis is an infectious inflammatory disease that destroys the tooth-supporting tissues. It is initiated by complex subgingival biofilms, triggering an inflammatory response by the juxtaposed gingival tissue. The range of transcriptional events initiated in gingiva following biofilm challenge is not fully elucidated. By employing gene microarray technology, this study aimed to characterize the overall transcriptional changes (> 2-fold regulation) of cultured human gingival fibroblasts (GF) in response to a 10-species *in vitro* subgingival biofilm model (BF), over a challenge period of 6 h. The relative involvement of the three "red complex" species in these transcriptional events was evaluated by omitting these species from the biofilm composition (BF-RC). When compared to the unchallenged control, challenge with BF and BF-RC differentially regulated 386 and 428 genes, respectively, with an overlap of 52%-75%. Interestingly, the expression of only 3 genes was significantly different between the BF and BF-RC challenged groups. There was also a strong overlap of the affected signaling pathways and gene ontology processes. These signaling pathways involved primarily the immune response, and included toll-like receptors (TLR), interleukin (IL)-1, IL-17, and heat shock protein (HSP) 60 and HSP 70. In conclusion, subgingival biofilms elicited a large number of transcriptional changes in GF, while the presence of the "red complex" in the biofilm did not yield any substantial differences. These findings show a uniform "non-specific" transcriptional response of host cells to subgingival biofilms, and denote that redundancies may exist in the virulence properties of individual bacterial species within a polymicrobial biofilm community.

Introduction

Periodontitis is a common oral infection characterised by the inflammatory-induced destruction of the tooth-supporting (periodontal) tissues. It is characterized by an excessive chronic inflammatory response of the resident tissues. This process will eventually lead to tooth loss, if the inflammation remains unresolved and the disease untreated. The inflammation is initiated by bacteria forming complex biofilm communities on the tooth surface and embedded into a dense extracellular matrix of both bacterial and host components (Marsh 2005). Failure to eliminate the biofilms from the tooth surface will eventually result in subgingival growth of the biofilm. This will interact with the juxtaposed gingival tissue, which may respond by a cascade of tissue-destructive inflammatory reactions (Schenkein 2006). Although more than 700 species can colonize in the oral cavity (Aas and others 2005) and approximately 500 of these species may be detected in subgingival biofilms (Paster and others 2001), only a limited number is associated with periodontitis (Papapanou and others 2009). More evidently implicated in the etiology of the disease are *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, also designated as the "red complex" species (Socransky and others 1998). Yet in light of recent evidence, polymicrobial synergy and dysbiosis is a detrimental factor for the initiation of periodontal disease, beyond the "red complex". In this respect, different members of the microbial community, or specific gene combinations, exhibit converging roles in eliciting the pathogenic mechanisms that govern the disease (Hajishengallis and others 2012; Hajishengallis and Lamont 2012).

The physiological role of the gingival tissue is to support the tooth and form a first line of defense against the microorganisms of the developing biofilms. The gingival epithelial layers are disrupted by the bacteria of the biofilm or their individual components. Consequently, it is possible for them to interact with the exposed gingival connective tissue, stimulating a cascade of inflammatory events that lead to periodontal tissue destruction.

Gingival fibroblasts (GF) are the major population of the gingival connective tissue. They are responsible for the synthesis and degradation of the extracellular matrix, and react to bacterial challenges by producing mediators of inflammation and bone resorption, as well as proteolytic enzymes (Belibasakis and Guggenheim 2011; Belibasakis and others 2011; Bostanci and others 2011). In doing so, they are crucial for regulating the homeostasis of the periodontal tissues in health and disease (Bartold and others 2000).

Transcriptomic analyses have been used in various models employing GF, for instance in order to compare the differences in gene expression patterns between GF and periodontal ligament cells (Han and Amar 2002) or gingival epithelial cells (Abiko and others 2004), or between healthy and periodontally diseased tissues (Wang and others 2003). In some experimental studies, gene microarray technologies have also been used to investigate the global transcriptional regulation in GF stimulated with inflammatory mediators, such as tumor necrosis factor (TNF)-α (Bage and others 2010; Davanian and others 2012a; Davanian and others 2012b). Alternatively, single bacterial species, such as *P. gingivalis*, have been used to challenge bone marrow stromal cells (Reddi and Belibasakis 2012), or aortic smooth muscle cells (Zhang and others 2013), and thereafter analysed for the occurring transcriptional responses. Nevertheless, no experimental studies are available which collectively investigate the transcriptional effects of multi-species biofilms, rather than single bacteria in planktonic state, on host cells. This approach is more relevant to the pathogenesis of periodontal diseases, as the etiological factor is polymicrobial biofilm. By employing a gene microarray platform, the present study aimed to characterise the global transcriptional profile of GF in response to a 10-species *in vitro* subgingival biofilm model. A further aim was to evaluate the relative involvement of the three "red complex" species, by excluding them from the biofilm composition and comparatively investigating the elicited effects.

Methods

In vitro biofilm model

The 10-species *in vitro* biofilm model used in this study, consisted of bacteria primarily identified subgingivally. These were namely *Campylobacter rectus* (OMZ 698), *Fusobacterium nucleatum* (OMZ 598), *Porphyromonas gingivalis* ATCC 33277^T (OMZ 925), *Prevotella intermedia* ATCC 25611^T (OMZ 278), *Tannerella forsythia* (OMZ 1047), *Treponema denticola* ATCC 35405^T (OMZ 661), *Veillonella dispar* ATCC 17748^T(OMZ 493), *Actinomyces oris* (OMZ 745), *Streptococcus anginosus* (OMZ 871), and *Streptococcus oralis* SK 248 (OMZ 607). This biofilm variant was designated as "BF". A 7-species version of this biofilm was also grown, in the absence of strain *P. gingivalis*, *T. denticola* and *T. forsythia*. This biofilm variant was designated as "BF-RC". The biofilms were generated as previously described (Ammann and others 2013; Belibasakis and others 2013a; Belibasakis and others 2013b). Briefly, the bacteria were grown in 24-well cell culture plates on sintered hydroxyapatite discs, resembling a natural tooth surface. To achieve pellicle formation, these surfaces were pre-conditioned for 4 h with 800 µl foetal bovine serum (FBS), which was diluted 1:1 in 25 % of sterile physiological saline. To initiate biofilm formation, the hydroxyapatite discs were covered with 1.6 ml of growth medium consisting of 70 % FBS (diluted 1:10) and 30% modified fluid universal medium (mFUM) containing 0.3 % glucose, and 200 µl of a bacterial cell suspension containing equal volumes and densities from each strain. After 16 h of anaerobic incubation at 37 °C, the discs were transferred into wells with fresh medium, and incubated for further 48 h, in anaerobic atmosphere. During this time-period, the discs were "dip-washed" three times daily in physiological saline and were given fresh medium once daily. After a total 64 h of incubation, each biofilm-carrying hydroxyapatite disc was ready for placement onto the GF cultures, as described in the next section, for a challenge period of 6 h. After this time, the discs were collected and analysis of

the composition of each individual species was performed by bacterial cell culture, or by immunofluorescence (IF), or by fluorescent *in situ* hybridization (FISH), as previously described (Belibasakis and others 2013a). The statistical significance of differences in bacterial numbers between BF and BF-RC was calculated by the Student's t-test ($P = 0.05$).

Establishment of GF cell cultures and challenge with the biofilm

Primary human GF were established as previously described (Belibasakis and others 2002; Belibasakis and others 2011; Bostanci and others 2011). The cells were passaged and cultured in Minimum Essential Medium Alpha (Gibco/Invitrogen, Lucerne, Switzerland), supplemented with 5 % heat-inactivated FBS, 50 Units/ml of penicillin, and 50 μg/ml streptomycin (Sigma-Aldrich, Buchs, Switzerland). For the experiments, cells at passage 4 were seeded at a concentration of 5 x 10^5 cells/cm^2 in antibiotics-free culture medium, supplemented with 5% FBS, and were allowed to attach overnight. Thereafter, one hydroxyapatite disc was placed carefully into each cell culture well, with the biofilm-coated surface facing towards the GF monolayer. A plastic ring was delicately placed into the wells in advance, in order to ensure a distance of 1 mm between the disc and the underlying cells, hence allowing for fluid flow, as already described for this host-biofilm interaction model (Belibasakis and others 2013a). As controls, pellicle-coated hydroxyapatite discs that did not contain biofilms were used. The experimental challenge with the biofilm lasted 6 h. Upon completion of this period, the hydroxyapatite discs were removed from the wells and the GF monolayers were processed for transcriptomic analysis, as described in the next section. The cell-free culture supernatants were collected and potential cytotoxicity was evaluated by measuring the extracellularly released lactate dehydrogenase (LDH) activity by the CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega, Mannheim, Germany), according to the manufacturer's instructions. Each of the three experimental groups was represented in four independent biological replicates.

RNA extraction and cRNA preparation

Upon completion of the experiments, after 6 h of challenge, the culture supernatants were removed and the cell monolayers were washed twice in PBS, before being lysed. Then, total RNA was extracted by the RNeasy Mini Kit (QIAGEN, Hombrechtikon, Switzerland), and eluted in RNase-free water. The concentration of the eluted total RNA was measured by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Reinach, Switzerland). For the further processing on the microarray template, the RNA concentration used was 100 ng/µl, in a total volume of 20 µl. The following procedures were performed at the microarray core facilities of the Functional Genomic Center Zürich (FGCZ), Switzerland.

The quality of the isolated RNA was determined with a NanoDrop 1000 spectophotometer and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). The 260 nm/280 nm ratio of the processed samples was in the range of 1.8–2.1, and the 28S/18S ratio in the range of 1.5–2.0. Total RNA (600 ng) was then reverse-transcribed into double-stranded cDNA in presence of RNA poly-A controls, RNA Spike-In Kit, One-Color (Agilent, Waldbronn, Germany). The double-stranded cDNA was then *in vitro* transcribed in presence of Cy3-labelled nucleotides using a Low RNA Input Linear Amp Kit+Cy dye, one-color kit (Agilent, Waldbronn, Germany). The Cy3-cRNA was purified using an RNeasy mini kit (QIAGEN, Hombrechtikon, Switzerland) and its quality and quantity were determined using NanoDrop 1000 and Bioanalyzer 2100. Samples with a total cRNA higher than 2 µg and a dye incorporation rate between 9-20 pmol/µg were considered further for hybridization.

Array hybridization, data processing and analysis

The Cy3-labeled cRNA samples (1.65 µg) were mixed with an Agilent Blocking Solution, subsequently randomly fragmented to 100-200 bp at 65°C with Fragmentation Buffer, and re-suspended in hybridization buffer using a Gene Expression Hybridization Kit (Agilent,

Waldbronn, Germany). Target cRNA Samples (100µl) were hybridized onto Human Gene Expression 4x44k v2 Microarray Kits (G4845A- Agilent, Waldbronn, Germany) for 17 h at 65°C. Arrays were then washed using Agilent GE Wash Buffers 1 and 2, according to the manufacturer's instructions (One-Color Microarray-Based Gene Expression Analysis Manual). An Agilent Microarray Scanner was used to measure the fluorescent intensity emitted by the labeled targets.

A total of 34,183 individual probes were printed for this array. The raw data processing was performed using the Agilent Scan Control and the Agilent Feature Extraction Software Version 10. Quality control measures were considered before performing the statistical analysis. These included, inspection of the array hybridization pattern (absence of scratches, bubbles, areas of non-hybridization), proper grid alignment, performance of the spike in controls (linear dynamic range between 5 orders of magnitude) and number of green feature non uniformity outliers (below 100 for all samples). The data was then imported onto the B-Fabric platform (Functional Genomics Center Zürich) for further transcriptomic analysis. In pairwise comparisons between the three groups, the Student's t-test was used to calculate the significance of the differences. A log2 ratio >1, with a *P* value <0.01 was considered as a true regulation. The quantified data with the list of gene accession numbers and their corresponding log2 ratio of regulation were imported into the web-based open access MetaCore^{TM} software (version 6.16 build 63671, Thomson Reuters, St. Joseph, MI, USA) for enrichment analysis of pathway maps and Gene Ontology (GO) processes, ranked based on the statistical significance (*P* value) of the uploaded data set.

Results

The bacterial composition of the biofilm was investigated following 6 h of co-culture with the GF (Table 1). It was found that there were no statistically significant differences between BF

and BF-RC in the numeric composition of each individual species, with the exception of the *P. gingivalis*, *T. forsythia* and *T. denticola*, which were omitted in first place from BF-RC. The potential cytotoxicity caused by either biofilm on the GF cultures was then investigated, after 6 h of challenge. No statistically significant differences were observed in the levels of extracellularely released LDH in BF or BF-RC, compared to the control (data not shown). Therefore, further transcriptomic analysis was possible in this experimental system.

The expression of a total of 34,183 individual transcripts was assayed by this microarray. Pairwise comparisons were performed between the three study groups. To narrow-down the frame of reference of these comparisons, a cut-off threshold of 2-fold regulation (log2 ratio > 1) was chosen, either this referred to up-regulation or down-regulation. Transcripts with a designated accession number, Entrez gene ID and Gene Symbol were taken into consideration for further analysis. Some of the regulated transcripts were represented more than once in this microarray platform. The overview of the numbers of regulated transcripts per group comparison is provided in Table 2. Firstly, comparison was made between the BF and the control group. It was found that 419 transcripts were regulated in response to BF challenge, involving 230 up-regulated and 189 down-regulated transcripts (Table 2). The comparison between the BF-RC and the control group revealed that 460 transcripts were regulated, which involved 217 up-regulated and 243 down-regulated transcripts (Table 2A). Strikingly, the direct comparison between the BF-RC and BF groups revealed that only 3 transcripts were differentially regulated (Table 2A), including nuclear receptor subfamily 1, group I, member 3 (*NR1I3*), CD86 molecule (*CD86*), and an exocyst-like pseudogene. The full list of regulated transcript is provided as supplementary material (Supplementary Table).

Some of the measured transcripts encoded for the same unique genes, as defined by accession number and gene name. Hence, when the number of unique genes, rather than transcripts was considered, 386 genes were significantly regulated by BF and 428 by BF-RC,

compared to the control. The degree of overlap of the regulated genes between these two-group comparisons was further analyzed. It was found that, in relation to the control group, the BF and BF-RC challenge commonly up-regulated 153 transcripts and down-regulated 119 transcripts, respectively (Table 2B).

It was the further aimed to analyse the pathway maps and GO processes regulated in response to the two biofilm variants. Starting with the pathway maps, 9/10 of the top regulated pathways in GF were identical in response to either BF or BF-RC (Table 3). These were predominantly related to the regulation of immune response and included the signalling pathways via toll-like receptors (TLRs), interleukin (IL)-1, IL-17, heat shock proteins (HSP) 60 and 70, High-Mobility Group Box 1 (HMGB1)/ Receptor for Advanced Glycation End-products (RAGE). Hence, a high similarity in the regulated pathways was evident in response to either biofilm challenge, irrespective of the presence or absence of the "red complex" species. Only exceptions were the IL-33 signaling pathway, which was present in the top-10 pathways regulated by BF-RC, and the Protein kinase RNA-activated (PKR) pathway associated with stress-induced cell response, which was present in the top-10 pathways regulated by BF.

An enrichment analysis was also performed with regards to the regulated GO processes (Table 4). This revealed that 8/10 of the top regulated GO processes in GF were common in response to either BF (Table 4A) or BF-RC (Table 4B), as compared to the control. All included the regulation of cellular and macromolecular metabolic and biosynthetic processes. The number of regulated genes per GO process was at a very close range between the two comparisons, with no more than 3 differentially regulated genes. The main observable difference was the regulation of DNA-dependent transcription and RNA biosynthetic processes, in response to BF-RC. Notably, these two GO processes appeared only at the end of the top-10 list.

Discussion

The present study employed a gene microarray platform to characterize the transcriptomic responses of human GF against an *in vitro* subgingival biofilm. Within the aims was the evaluation of the differential effects of the three "red complex" species, by selectively excluding them from the biofilm composition. The microbial composition of the remaining species yielded no significant differences between two biofilm variants, and any differences were less than 1-log. There was a narrow difference in the number of significantly regulated genes in response to either of the two biofilms (386 versus 428), with an overlap of 73-77% and 52-68% up-regulated and down-regulated genes, respectively. There was also a very high overlap of the top-regulated signalling pathways and GO processes. The affected pathways involve primarily the innate immune responses, such as the regulation of signalling for IL-1 and IL-17 cytokines, HSP 60 and 70, and TLRs. IL-33 appeared also in the top-10 list of the pathways regulated by BF-RC challenge. This may not be surprising, as IL-33 is also a member of the IL-1 family of cytokines (Pei and others 2014). This suggests a uniform global transcriptional response of GF to polymicrobial biofilm challenge, irrespective of the presence or absence of the "red-complex" from the composition. These findings are well in line with documented host immune responses to periodontal pathogens. It has been shown earlier that lower protein concentrations of BF supernatants (up-to 30 μg/ml) increased the expression of IL-1β and IL-18 (both IL-1 cytokines) in GF, while higher concentrations (300 μg/ml) caused a down-regulation of these genes, accompanied by a down-regulation of the Nod-like receptor (NLR) family inflammasome NLRP3, responsible for IL-1 cytokine secretion (Bostanci and others 2011). Omitting *P. gingivalis* from the biofilm composition, only partially rescued this effect on IL-1β and NLRP3 elicited by BF (Belibasakis and others 2013a). Although there is collective evidence that subgingival biofilms regulate IL-1 signaling in GF, the present study demonstrates further that the involvement of the "red complex" species is not crucial for this.

Earlier studies used single bacterial species to investigate the full range of transcriptional events in a given cell type. For instance, in another microarray platform, *P. gingivalis* alone was shown to regulate in bone marrow stromal cells genes associated with inhibition of cell cycle, induction of apoptosis and loss of structural integrity at 6 h, whereas at 24 h this response was characterised by induction of chemokines, cytokines and mediators of connective tissue and bone destruction, marking a deregulated homeostatic function (Reddi and Belibasakis 2012). It has also been shown that *F. nucleatum* and *P. gingivalis* regulate the expression of the NF-kB pathway, including TNF-α, IL-1β, IL-8, and several cytokeratin gene family members, in oral epithelial cells. Nevertheless, there were trends of differential gene expression between the two bacterial species, with *F. nucleatum* inducing more transcriptional changes than *P. gingivalis* after 24 h, and only 91 genes commonly regulated in response to both pathogens (Milward and others 2007). In another model, *F. nucleatum* and *S. gordonii* perturbed the gingival epithelial cell transcriptional responses to a lesser extent than *P. gingivalis* or *A. actinomycetemcomitans*, indicating that a greater degree of host adaptation to commensal species, rather than putative pathogens (Handfield and others 2005). Based on a follow-up of these findings, it was also evident that TLR pathways ultimately converge on cytokine gene expression (Hasegawa and others 2007).

Transcriptional profiling data is also available from *ex vivo* samples obtained from patients with periodontal disease. Using a focused DNA microarray, it was found that human GF obtained from inflamed gingival tissues expressed higher levels of IL-1 cytokines, IL-6, IL-8, TNF-α, CD14, TLR-2 and TLR-4, than ones obtained from non-inflamed clinical healthy tissues (Wang and others 2003). Although the microbiological profile of these tissue sites is not clear, the findings of this earlier study are in agreement with the present work, whereby the "subgingival" *in vitro* biofilm regulated the IL-1, TLR-2 and TLR-4 signaling pathways. Of parallel interest is also the finding that oral neutrophils obtained from periodontal disease patients display an altered transcriptomic profile, which is characterized

by up-regulation of pro-survival, and down-regulation of pro-apoptotic genes. This may be linked to the longevity of the neutrophil inflammatory response periodontitis (Lakschevitz and others 2013). The host transcriptional profiles were examined in a murine calvarial model of inflammation and bone resorption, following mono-species infection with *T. forsythia*, *T. denticola* or *P. gingivalis*. The significantly affected biological pathways included leukocyte transendothelial migration, cell adhesion molecules related to the immune system, extracellular matrix–receptor interaction, adherens junction, and antigen processing and presentation (Bakthavatchalu and others 2010a; Bakthavatchalu and others 2010b; Meka and others 2010). When this model was adapted for polymicrobial infection that included all three "red complex" species, it was shown that similar biological pathways were regulated as in the case of the mono-infection, but involved additionally cytokine and chemokine-associated pathways (Bakthavatchalu and others 2011).

The most striking finding of the present study was revealed after the direct comparison of the transcriptional effects between BF and BF-RC groups. Among thousands of transcripts, only three genes proved to be were differentially regulated. These included *CD86* and *NR1I3*. CD86 is a receptor essential for T-cell responses, which can also be expressed by several non-lymphoid cells (Romero-Tlalolini and others 2013), including oral epithelial cells in response to *F. nucleatum* (Han and others 2003). *NR1I3* encodes for the constitutive androstane receptor (CAR), a member of the nuclear steroid/thyroid hormone receptor superfamily that regulates hepatic metabolism and detoxification (Zelko and Negishi 2000). Novel functions of CAR include the metabolic modulation of glucose, lipids, but also cell-cell communication, regulation of the cell cycle, and chemical carcinogenesis (Molnar and others 2013). It is not clear at this stage if the highly selective regulation of these genes by the "red complex" is of any biological significance for the mechanisms underlying the pathogenesis of periodontitis.

It is of further interest that the data yielded by the present *in vitro* study is paralleling clinical findings deriving from the transcriptomic analysis of gingival tissues obtained from

patients. A comparison between the global gene expression signatures in healthy and diseased gingival tissues indicated more than 12,000 differentially expressed transcripts, which were identified within 61 different GO processes (Demmer and others 2008). However, when the gingival transcriptional profiles were compared between the chronic and aggressive forms of periodontitis, only limited differences were revealed, suggesting limited molecular dissimilarities between the two types of lesions (Kebschull and others 2013). Nevertheless, both commonalities and differences can exist among tissue gene expression patterns, according to the subgingival microbial profile of the periodontal pocket (Papapanou and others 2009). It should also be noted that deep sequencing reveals great inter-individual variability in the microbial composition of subgingival biofilms in periodontal disease (Ge and others 2013, Schwarzberg and others 2014), spanning beyond the "red complex"

In conclusion, a large number of transcriptional changes are activated in GF in response to subgingival biofilms. These are primarily associated with the innate immune response. The presence of the three "red complex" species as part of the multi-species biofilm did not yield meaningful differential responses, at least not at the transcriptional level. Collectively, these findings denote that subgingival biofilms can cause a uniform "non-specific" transcriptional response by host cells. Overlaps or redundancies may exist in the virulence of each individual species within the biofilm community, in line with the model of polymicrobial synergy and dysbiosis for periodontitis (Hajishengallis and Lamont 2012).

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References

Aas, J.A., Paster, B.J., Stokes, L.N., Olsen, I., and Dewhirst, F.E. (2005) Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol* **43**: 5721-32.

Abiko, Y., Hiratsuka, K., Kiyama-Kishikawa, M., Tsushima, K., Ohta, M., and Sasahara, H. (2004) Profiling of differentially expressed genes in human gingival epithelial cells and fibroblasts by DNA microarray. *J Oral Sci* **46**: 19-24.

Ammann, T.W., Belibasakis, G.N., and Thurnheer, T. (2013) Impact of early colonizers on in vitro subgingival biofilm formation. *PLoS One* **8**: e83090.

Bage, T., Lindberg, J., Lundeberg, J., Modeer, T., and Yucel-Lindberg, T. (2010) Signal pathways JNK and NF-kappaB, identified by global gene expression profiling, are involved in regulation of TNFalpha-induced mPGES-1 and COX-2 expression in gingival fibroblasts. *BMC Genomics* **11**: 241.

Bakthavatchalu, V., Meka, A., Mans, J.J., Sathishkumar, S., Lopez, M.C., Bhattacharyya, I., Boyce, B.F., Baker, H.V., Lamont, R.J., Ebersole, J.L., and Kesavalu, L. (2011) Polymicrobial periodontal pathogen transcriptomes in calvarial bone and soft tissue. *Mol Oral Microbiol* **26**: 303-20.

Bakthavatchalu, V., Meka, A., Sathishkumar, S., Lopez, M.C., Bhattacharyya, I., Boyce, B.F., Mans, J.J., Lamont, R.J., Baker, H.V., Ebersole, J.L., and Kesavalu, L. (2010a) Tannerella forsythia infection-induced calvarial bone and soft tissue transcriptional profiles. *Mol Oral Microbiol* **25**: 317-30.

Bakthavatchalu, V., Meka, A., Sathishkumar, S., Lopez, M.C., Verma, R.K., Wallet, S.M., Bhattacharyya, I., Boyce, B.F., Mans, J.J., Lamont, R.J., Baker, H.V., Ebersole, J.L.,

and Kesavalu, L. (2010b) Molecular characterization of Treponema denticola infection-induced bone and soft tissue transcriptional profiles. *Mol Oral Microbiol* **25**: 260-74.

Bartold, P.M., Walsh, L.J., and Narayanan, A.S. (2000) Molecular and cell biology of the gingiva. *Periodontol 2000* **24**: 28-55.

Belibasakis, G., Johansson, A., Wang, Y., Claesson, R., Chen, C., Asikainen, S., and Kalfas, S. (2002) Inhibited proliferation of human periodontal ligament cells and gingival fibroblasts by Actinobacillus actinomycetemcomitans: involvement of the cytolethal distending toxin. *Eur J Oral Sci* **110**: 366-73.

Belibasakis, G.N. and Guggenheim, B. (2011) Induction of prostaglandin E(2) and interleukin-6 in gingival fibroblasts by oral biofilms. *FEMS Immunol Med Microbiol* **63**: 381-6.

Belibasakis, G.N., Guggenheim, B., and Bostanci, N. (2013a) Down-regulation of NLRP3 inflammasome in gingival fibroblasts by subgingival biofilms: involvement of Porphyromonas gingivalis. *Innate Immun* **19**: 3-9.

Belibasakis, G.N., Meier, A., Guggenheim, B., and Bostanci, N. (2011) The RANKL-OPG system is differentially regulated by supragingival and subgingival biofilm supernatants. *Cytokine* **55**: 98-103.

Belibasakis, G.N., Thurnheer, T., and Bostanci, N. (2013b) Interleukin-8 responses of multi-layer gingival epithelia to subgingival biofilms: role of the "red complex" species. *PLoS One* **8**: e81581.

Bostanci, N., Meier, A., Guggenheim, B., and Belibasakis, G.N. (2011) Regulation of NLRP3 and AIM2 inflammasome gene expression levels in gingival fibroblasts by oral biofilms. *Cell Immunol* **270**: 88-93.

Davanian, H., Bage, T., Lindberg, J., Lundeberg, J., Concha, H.Q., Sallberg Chen, M., and Yucel-Lindberg, T. (2012a) Signaling pathways involved in the regulation of

TNFalpha-induced toll-like receptor 2 expression in human gingival fibroblasts. *Cytokine* **57**: 406-16.

Davanian, H., Stranneheim, H., Bage, T., Lagervall, M., Jansson, L., Lundeberg, J., and Yucel-Lindberg, T. (2012b) Gene expression profiles in paired gingival biopsies from periodontitis-affected and healthy tissues revealed by massively parallel sequencing. *PLoS One* **7**: e46440.

Demmer, R.T., Behle, J.H., Wolf, D.L., Handfield, M., Kebschull, M., Celenti, R., Pavlidis, P., and Papapanou, P.N. (2008) Transcriptomes in healthy and diseased gingival tissues. *J Periodontol* **79**: 2112-24.

Ge, X., Rodriguez, R., Trinh, M., Gunsolley, J., Xu, P. (2013) Oral microbiome of deep and shallow dental pockets in chronic periodontitis. *PLoS One* **8**: e65520.

Hajishengallis, G., Krauss, J.L., Liang, S., McIntosh, M.L., and Lambris, J.D. (2012) Pathogenic microbes and community service through manipulation of innate immunity. *Adv Exp Med Biol* **946**: 69-85.

Hajishengallis, G. and Lamont, R.J. (2012) Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol* **27**: 409-19.

Han, D.C., Huang, G.T., Lin, L.M., Warner, N.A., Gim, J.S., and Jewett, A. (2003) Expression of MHC Class II, CD70, CD80, CD86 and pro-inflammatory cytokines is differentially regulated in oral epithelial cells following bacterial challenge. *Oral Microbiol Immunol* **18**: 350-8.

Han, X. and Amar, S. (2002) Identification of genes differentially expressed in cultured human periodontal ligament fibroblasts vs. human gingival fibroblasts by DNA microarray analysis. *J Dent Res* **81**: 399-405.

Handfield, M., Mans, J.J., Zheng, G., Lopez, M.C., Mao, S., Progulske-Fox, A., Narasimhan, G., Baker, H.V., and Lamont, R.J. (2005) Distinct transcriptional profiles characterize oral epithelium-microbiota interactions. *Cell Microbiol* **7**: 811-23.

Hasegawa, Y., Mans, J.J., Mao, S., Lopez, M.C., Baker, H.V., Handfield, M., and Lamont, R.J. (2007) Gingival epithelial cell transcriptional responses to commensal and opportunistic oral microbial species. *Infect Immun* **75**: 2540-7.

Kebschull, M., Guarnieri, P., Demmer, R.T., Boulesteix, A.L., Pavlidis, P., and Papapanou, P.N. (2013) Molecular differences between chronic and aggressive periodontitis. *J Dent Res* **92**: 1081-8.

Lakschevitz, F.S., Aboodi, G.M., and Glogauer, M. (2013) Oral neutrophil transcriptome changes result in a pro-survival phenotype in periodontal diseases. *PLoS One* **8**: e68983.

Marsh, P.D. (2005) Dental plaque: biological significance of a biofilm and community life-style. *J Clin Periodontol* **32 Suppl 6**: 7-15.

Meka, A., Bakthavatchalu, V., Sathishkumar, S., Lopez, M.C., Verma, R.K., Wallet, S.M., Bhattacharyya, I., Boyce, B.F., Handfield, M., Lamont, R.J., Baker, H.V., Ebersole, J.L., and Kesavalu, L. (2010) Porphyromonas gingivalis infection-induced tissue and bone transcriptional profiles. *Mol Oral Microbiol* **25**: 61-74.

Milward, M.R., Chapple, I.L., Wright, H.J., Millard, J.L., Matthews, J.B., and Cooper, P.R. (2007) Differential activation of NF-kappaB and gene expression in oral epithelial cells by periodontal pathogens. *Clin Exp Immunol* **148**: 307-24.

Molnar, F., Kublbeck, J., Jyrkkarinne, J., Prantner, V., and Honkakoski, P. (2013) An update on the constitutive androstane receptor (CAR). *Drug Metabol Drug Interact* **28**: 79-93.

Papapanou, P.N., Behle, J.H., Kebschull, M., Celenti, R., Wolf, D.L., Handfield, M., Pavlidis, P., and Demmer, R.T. (2009) Subgingival bacterial colonization profiles correlate with gingival tissue gene expression. *BMC Microbiol* **9**: 221.

Paster, B.J., Boches, S.K., Galvin, J.L., Ericson, R.E., Lau, C.N., Levanos, V.A., Sahasrabudhe, A., and Dewhirst, F.E. (2001) Bacterial diversity in human subgingival plaque. *J Bacteriol* **183**: 3770-83.

Pei, C., Barbour, M., Fairlie-Clarke, K.J., Allan, D., Mu, R., and Jiang, H.R. (2014) Emerging role of interleukin-33 in autoimmune diseases. *Immunology* **141**: 9-17.

Reddi, D. and Belibasakis, G.N. (2012) Transcriptional profiling of bone marrow stromal cells in response to Porphyromonas gingivalis secreted products. *PLoS One* **7**: e43899.

Romero-Tlalolini, M.A., Chavez Olmos, P., and Garrido, E. (2013) Differential DNA methylation patterns in the CD86 gene controls its constitutive expression in keratinocytes. *Biochem Biophys Res Commun* **438**: 54-60.

Schenkein, H.A. (2006) Host responses in maintaining periodontal health and determining periodontal disease. *Periodontol 2000* **40**: 77-93.

Schwarzberg, K., Le, R., Bharti, B., Lindsay, S., Casaburi, G., Salvatore, F., Saber, M.H., Alonaizan, F., Slots, J., Gottlieb, R.A., Caporaso, J.G., Kelley, S.T. (2014) The personal human oral microbiome obscures the effects of treatment on periodontal disease. *PLoS One* **9**: e86708.

Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C., and Kent, R.L., Jr. (1998) Microbial complexes in subgingival plaque. *J Clin Periodontol* **25**: 134-44.

Wang, P.L., Ohura, K., Fujii, T., Oido-Mori, M., Kowashi, Y., Kikuchi, M., Suetsugu, Y., and Tanaka, J. (2003) DNA microarray analysis of human gingival fibroblasts from healthy and inflammatory gingival tissues. *Biochem Biophys Res Commun* **305**: 970-3.

Zelko, I. and Negishi, M. (2000) Phenobarbital-elicited activation of nuclear receptor CAR in induction of cytochrome P450 genes. *Biochem Biophys Res Commun* **277**: 1-6.

Zhang, B., Elmabsout, A.A., Khalaf, H., Basic, V.T., Jayaprakash, K., Kruse, R., Bengtsson, T., and Sirsjo, A. (2013) The periodontal pathogen Porphyromonas gingivalis changes

the gene expression in vascular smooth muscle cells involving the TGFbeta/Notch signalling pathway and increased cell proliferation. *BMC Genomics* **14**: 770.

Tables

Table 1. Bacterial composition of the subgingival biofilms after 6 h of co-culture with GF

	BF	***BF-RC***
A. oris	6.7 E6 ± 3.8 E6	7.0 E6 ± 4.1 E6
V. dispar	1.2 E8 ± 4.3 E7	7.8 E7 ± 1.7 E7
F. nucleatum	2.8 E8 ± 7.5 E7	2.0 E8 ± 1.1 E8
S. anginosus	2.0 E7 ± 3.0 E6	1.5 E7 ± 5.8 E6
S. oralis	1.3 E8 ± 4.9 E7	2.3 E8 ± 9.2 E7
P. intermedia	1.2 E8 ± 4.9 E7	8.0 E7 ± 3.5 E7
C. rectus (a)	2.7 E4 ± 1.6 E4	1.7 E4 ± 1.7 E4
P. gingivalis	9.3 E5 ± 3.1 E5	---
T. forsythia (b)	6.8 E5 ± 5.8 E4	---
T. denticola (c)	1.2 E6 ± 9.2 E5	---

The quantitative composition of the individual bacterial species in the biofilms after 6 h of co-culture with GF was defined by bacterial culture analysis (colony forming unit measurement), or FISH, or IF, as previously described (Belibasakis and others 2013a). (a) The detection and counting of *C. rectus* was performed by IF, using monoclonal antibody 212WR2. (b) The detection and counting of *T. forsythia* was performed by IF, using monoclonal antibody 103BF1.1 (c). The detection and counting of *T. denticola* was performed by FISH, using DNA probe TrepG1-679-Cy3 (5' to 3' sequence: GATTCCACCCCTACACTT). The data represents the bacterial mean counts ± SD from triplicate biofilm cultures. BF; 10-species biofilm challenged group, BF-RC; 7-species biofilm challenged group (in which the "red complex" was absent from the composition).

Table 2

A. Overview of significantly regulated transcripts between the three study groups

Group comparisons	***Total***	***Up-regulated***	***Down-regulated***
BF versus Control	419	230	189
BF-RC versus Control	460	217	243
BF-RC versus BF	3	0	3

The table presents the number of transcripts significantly (P<0.01) regulated more than 2-fold in between groups. Control; Un-challenged group, BF; 10-species biofilm challenged group, BF-RC; 7-species biofilm challenged group (in which the "red complex" was absent from the composition).

B. Overlap of regulated genes in response to BF and BF-RF

Group comparisons	***Total***	***Common up-regulated***	***Common down-regulated***
BF versus Control	386	153/211 (72.5 %)	119/175 (68.0 %)
BF-RC versus Control	428	153/199 (76.9 %)	119/229 (52.0 %)

The table presents the total number of genes regulated, as well as the overlap of regulated genes (percentage) by the two biofilms, in compared to the unchallenged control. Control; Un-challenged group, BF; 10-species biofilm challenged group, BF-RC; 7-species biofilm challenged group (in which the "red complex" was absent from the composition).

Table 3. Enrichment analysis by Pathway Maps

A. Comparison BF versus control

Pathway Map	***Regulated genes***	***P value***
1. Immune response: IL-1 signaling	15/44	1.063E-17
2. Immune response: HSP60 and HSP70/ TLR signaling	15/54	3.561E-16
3. Expression targets of tissue factor signaling in cancer	9/22	6.532E-12

4. Immune response: PKR in stress-induced cell response	12/57	1.363E-11
5. Immune response: IL-17 signaling	12/60	2.604E-11
6. Immune response: TLR2 and TLR4 signaling	11/57	2.749E-10
7. Immune response: TLR5, TLR7, TLR8 and TLR9 signaling	10/48	8.311E-10
8. Immune response: HMGB1/RAGE signaling	10/53	2.336E-09
9. Reproduction: GnRH signaling	11/72	3.773E-09
10. Immune response: MIF-mediated glucocorticoid regulation	7/22	1.266E-08

B. Comparison BF-RC versus Control

Pathway Map	***Regulated genes***	***P value***
1. Immune response: IL-1 signaling	13/44	1.346E-14
2. Immune response: HSP60 and HSP70/ TLR signaling	11/54	1.355E-10
3. Expression targets of Tissue factor signaling in cancer	8/22	2.972E-10
4. Immune response: IL-17 signaling	10/60	7.696E-09
5. Immune response: MIF-mediated glucocorticoid regulation	7/22	1.197E-08
6. Immune response: TLR5, TLR7, TLR8 and TLR9 signaling	9/48	1.512E-08
7. Immune response: HMGB1/RAGE signaling	9/53	3.770E-08
8. Reproduction: GnRH signaling	10/72	4.758E-08
9. Immune response: TLR2 and TLR4 signaling	9/57	7.304E-08
10. Immune response: IL-33 signaling	9/57	7.304E-08

The top-10 regulated MetaCore pathway maps are presented in response to the challenge with either the 10-species biofilm (BF), or the 7-species biofilm lacking the "red complex" (BF-RC). The number of regulated genes is given, in relation to the total number of genes available in the pathway, as well as the levels of significance of the regulation.

Table 4. Enrichment analysis by GO Processes

A. Comparison BF versus Control

GO Process	***Regulated genes***	***P value***
1. Cellular metabolic process	229/5957	1.277E-47
2. Primary metabolic process	225/5920	2.401E-45
3. Metabolic process	238/6785	3.795E-43
4. Nitrogen compound metabolic process	198/4772	5.128E-43
5. Macromolecule metabolic process	216/5674	1.482E-42
6. Biosynthetic process	192/4538	2.005E-42
7. Macromolecule biosynthetic process	186/4277	2.576E-42
8. Nucleobase-containing compound metabolic process	194/4657	5.090E-42
9. Cellular biosynthetic process	190/4486	7.320E-42
10. Cellular macromolecule biosynthetic process	182/4163	2.204E-41

B. Comparison BF-RC versus Control

GO Processes	***Regulated genes***	***P value***
1. Cellular metabolic process	226/5957	3.901E-42
2. Primary metabolic process	223/5920	1.083E-40
3. Nitrogen compound metabolic process	198/4772	3.142E-40
4. Macromolecule metabolic process	216/5674	1.941E-39
5. Nucleobase-containing compound metabolic process	194/4657	2.526E-39
6. Biosynthetic process	189/4538	7.055E-38
7. Metabolic process	235/6785	1.137E-37
8. Transcription, DNA-dependent	172/3833	1.490E-37
9. Cellular biosynthetic process	187/4486	2.264E-37
10. RNA biosynthetic process	172/3851	2.790E-37

The top-10 regulated MetaCore GO Processes are presented in response to the challenge with either the 10-species biofilm (BF), or the 7-species biofilm lacking the “red complex” (BF-RC). The number of regulated genes is given, in relation to the total number of genes available in the pathway, as well as the levels of significance of the regulation.